White Clover Morphology Changes with Stress Treatments

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ABSTRACT

The plasticity of white clover (*Trifolium repens* L.) results in changes in plant habit in response to different environmental stresses. The objective of this research was to characterize those morphological changes associated with plasticity in white clover clones derived from 'Osceola', 'Grassland Huia', and SRVR germplasm. Clones were exposed to treatments in the following two-way factorial design: no clipping or clipping on 7-d intervals and barrier to stolon rooting or no barrier. In a three-season greenhouse test, 90 clones were measured for leaf dry weight (DW), stolon DW, stolon length, root DW, and apex number. Generated parameters were herbage DW (leaf DW + stolon DW), biomass DW (root DW + herbage DW), leaf-to-stem ratio (leaf DW ÷ stolon DW), and herbage-to-root ratio (herbage DW ÷ root DW). Plant means were significantly reduced for all traits and ratios with repeated clipping. The rooting barrier significantly reduced apex number, root DW, and the leaf-to-stem ratio while increasing the stolon DW and the herbage-to-root ratio. The interaction of root barrier and repeated clipping was significant for apex number, root DW, and the herbage-to-root ratio. Near zero correlations for the same measurements made on the same clone grown with different stress treatments suggest genetic differences in the magnitude of plasticity. Path analysis further described changes in relationships among traits for plants exposed to different stresses. The magnitude of plasticity appeared to be a clone-specific phenomenon that may frustrate verifying gain with selection for progenies of selections evaluated in different environments.

White clover is an important forage legume in many temperate regions of the world (Frame and Newbould, 1986), particularly in mixed species pastures. White clover adaptability is attributed, in part, to genetic variability in the species (Burdon, 1980; Turkington and Harper, 1979). Perenniality of white clover depends on renewal of plant parts (Beinhart, 1963; Williams, 1987), and a recently established stand of white clover evolves from tap root growth to clonal growth in 1 to 3 yr (Brock and Tilbrook, 2000). Persistence of white clover ecotypes is positively associated with stolon density, dry matter yield, and internode length (Piano and Annicchiarico, 1995). When grazed, cyclic morphological changes include increasing stolon length during the spring with a maximum in June and July followed by a decline during the summer to a minimal length in October (Brink et al., 1998). Change in stolon density has been linked to plant habit so that the less branched, more viney plant habit has much less cyclic change in stolon density (Gibson et al., 1963). The rate of spreading of white clover is positively correlated with increased size of plant parts and reduced leaf density per unit area (Beinhart et al., 1963).

Defoliation through grazing or clipping is a management-controlled stress that affects plant persistence and productivity. In a pasture, grazing removes the leaf but the stolon is usually undisturbed (Williams and Hogland, 1978). The defoliation mobilizes energy stores in stolon and root to produce new leaves and other photosynthetic components (Kendall and Stringer, 1985).

White clover has a remarkable phenotypic plasticity which results in a nonheritable change in plant morphology in response to environment, season, and management or harvest (Brock et al., 1988; Solangaarachchi and Harper, 1989). In mixed pastures, plant morphology changes in response to interspecific competition from Kentucky bluegrass (*Poa pratensis* L.) (Atwood and Garber, 1942), perennial ryegrass (*Lolium perenne* L.) (Dijkstra and de Vos, 1972), and bermudagrass [*Cynodon dactylon* (L.) Pers.] (Brink and Rowe, 1993; Rowe and Brink, 1993). The white clover changes are more complex than consistent decreases in vigor, viability, or reproduction of the plant or stand (Duncan, 1994). Response to stress is more than a simple decrease in productivity.

White clover is commonly characterized by its leaf size (Williams, 1987). Leaf size has been related to growth habit (Eagles and Othman, 1986; Kang et al., 1995), field growth, or spread (Davies and Evans, 1982); grazing responses (Brink and Pederson, 1993; Brock and Tilbrook, 2000); and rate of regrowth with clipping (Wilman and Asiegbu, 1982b). Reducing the time between clippings has more dramatic and detrimental effects on large leaf types of white clover than on the small leaf types (Wilman and Asiegbu, 1982a; Grant and Barthram, 1991). For the white clover seedling, reducing the clipping frequency resulted in a quadratic increase of plant dry weight and this increase was greater for the large leaf white clover cultivars. Annual DM yield doubled or tripled when harvest interval changed from 7 to 28 d, but for the 48-d harvest interval the yields were comparable to the 7-d harvest interval (Brink, 1995). Clipping causes a greater reduction in stolon length, herbage yield (Wilman and Asiegbu, 1982b), and root carbohydrate content (Kang and Brink, 1995) of the large leaved than for the small leaved white clover. With defoliation, more carbohydrate is partitioned to the apex and less to the main stolon; however, the degree of partitioning between root and apex is cultivar dependent (Frankow-Lindberg, 1997). Defoliation of greenhouse plants has been shown to significantly reduce bud viability by 44% and similar reductions may occur in pastures (Newton and Hay, 1996).

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Abbreviations: DW, dry weight; SD, standard deviation.

Restriction of node rooting causes greater reduction in growth of nodes more distal to the last rooted node (Lotscher and Nosberger, 1996) and root development is greater for the remaining roots. In the field, frequent defoliation caused a 28% decrease in stolon dry matter, 82% decrease in total non-structural carbohydrates, and 60% decrease in node development. Defoliation effects seemed to mirror the effects of grass competition, but were much more severe (Lüscher et al., 2001). In the presence of dense grass competition, white clover stolons can be elevated above the soil surface so that stolon rooting is less frequent. Continuous and rotational grazing of white clover showed that Grassland Huia had greater stolon density than three cultivars with larger leaves, but stolon survival at the end of the growing season for four cultivars were not significantly different (Brink and Pederson, 1993).

The objective of this research was to quantify the morphological changes associated with plasticity for an array of white clover genotypes subjected to stresses of frequent clipping and a barrier to stolon rooting.

MATERIALS AND METHODS

The genetic materials evaluated were random selections from a field planted with cultivars Osceola and Grasslands Huia and the germplasm SRVR (Gibson et al., 1989). Osceola and Grasslands Huia are classified as large and medium-small leaved types, respectively, (Williams, 1987) and SRVR has a medium-large leaf (Gibson et al., 1989). Originally 300 plants of each white clover entry were space planted (1 m on centers) into a common bermudagrass sod that was grazed by cattle continuously for 12 mo at Leveck Animal Research Center, Mississippi State University on a Catalpa clay (fine, montmorillonitic, thermic Fluvaquentic Hapludoll). For each white clover entry, over thirty clones were randomly selected from the field of survivors and rooted cuttings were potted in the greenhouse. Plants were maintained in the greenhouse and cuttings were rooted as needed for three greenhouse tests. The greenhouse evaluations were in the Spring (2 April–6 June), Summer (7 June–14 September), and winter (22 October–2 February). For each greenhouse evaluation, 12 cuttings of 10 different plants from each cultivar and germplasm were rooted in a mist chamber and 40 d later even-sized cuttings were planted into flats (48 by 25 by 4.5 cm) that contained a 1:1 mixture by volume of washed sand and artificial soil mix (Pro-Mix media, Premier Brands, Inc., New Rochelle, NY). The soil mixture was amended to a pH of 6.0 and field equivalent levels of 100 kg extractable P ha^{-1} and 250 kg exchangeable K ha^{-1}. Cuttings were inoculated with *Rhizobium leguminosarum* var *trifolii* Jordan and during the growing season 2 L of modified Hoagland's nutrient solution was applied to each flat. In the greenhouse, flats were arranged in a randomized complete block design with three replications. Each of the three seasonal evaluations used a different collection of white clover clones.

The treatments, applied in a factorial arrangement, were removal of leaf mass on 7-d intervals or no leaf removal and a soil cover as a barrier to stolon root development or no barrier to root development. The frequent clipping was an imitation of stress caused by continuous grazing and the rooting barrier imitated aerial growth of clover stolons in a competitive grass sward. Soil surface of half of the flats was covered with landscaping fabric (Patrician Products Inc., Hicksville, New York) which was a physical barrier (no herbicide) to stolon root development and the other half of the flats were not covered. In half of the flats, plants were subjected to the clipping treatment where all leaf and petiole material was removed four times on 7-d intervals beginning 33 d after transplanting. At the termination of the test, plants were washed of soil and stored at 4°C. Roots, stolons, and leaves were separated for drying and weighing, and measurements were made of the number of apexes (stolon tips ≥1 cm), and the total length of stolons (cm). The plant parts (leaf, root, and stolon) of each plant were dried at 60°C to get individual DW. Other parameters calculated from the dry weights were herbage DW (leaf DW + stolon DW), biomass DW (herbage DW + root DW), leaf-to-stem ratio (leaf DW ÷ stolon DW, since the clover stem is a stolon), and herbage-to-root ratio (herbage DW ÷ by root DW).

Data analysis used SAS programs (SAS Institute, 1989, Cary, NC). Path analysis was utilized to partition the correlation matrix into causal and spurious effects and then the causal effect was partitioned into direct (path coefficient) and indirect effects (correlations) (Ullman, 1996). Path analysis is a multivariate, multiple regression procedure used to examine perceived relationships of interest. As with other multiple regression procedures, the choice of dependent and independent variable is defined as a function of what appears scientifically reasonable and what relationships are of interest. In this case, path analysis quantifies causal relationships among measured plant parts to elucidate responses to clipping and rooting barrier treatments. The path coefficient is the regression coefficient from a regression on standardized variables and is also known as β weights in multiple regression analysis. Modeling assumptions for path analysis are those of linear regression and correlation analysis: additive, linear relationships. Path analysis was performed separately for each of the four treatment combinations.

In this data set, the spring yields were several times greater than those of the other seasons and a regression on the complete data set would be dominated by the responses in the spring to the exclusion of information in the other seasons. Under the assumption that information from each season is equally informative for characterization of changes in plant morphology, the data set for each season were standardized (variables had means of zero and variances of 1.0) separately. The three standardized data sets were then combined for path analysis. Thus relationships shown with this path analysis reflects responses generalized for three greenhouse environments.

RESULTS AND DISCUSSION

Confidence in conclusions about white clover growth and morphological responses to the treatments is dependent on both the array of genotypes evaluated and the range of background environments utilized in the evaluation. The self-sterility of white clover ensures some genetic heterogeneity in the individual plant, but to ensure broad genetic sampling, a total of 30 plants were randomly selected from each of two cultivars and a germplasm that had obvious differences in growth habit. One of the cultivars, Grasslands Huia, has a viney type of growth and the other two are nonviney types and each entry had a different leaf size. However, the array of genotypes included was limited to those genotypes surviving two years of continuous grazing and grass competition in a central Mississippi pasture. As reviewed earlier, white clover stands evolve from a relatively brief tap root growth phase to a clonal growth phase. The

Table 1. Trait means of individual plants from white clover cultivars Grasslands Huia and Osceola and germplasm SRVR.

Trait	White clover entry		
	Grasslands huia	Osceola	SRVR
Stolon length (cm)	568	487	479
Stolon DW (g)	3.90	6.52	6.17
Apex number	66.3	45.5	50.9
Leaf DW (g)	5.16	8.54	7.89
Root DW (g)	2.81	3.68	3.90
Herbage DW (g)	9.06	15.06	14.05
Biomass DW (g)	11.87	18.74	17.95
Leaf-to-stem ratio	1.32	1.31	1.28
Herbage-to-root ratio	3.22	4.09	3.60

use of cloned materials in this study may approximate the field clonal growth phase but is unlikely to be applicable to the tap root growth phase.

Differences in morphology of these white clover entries are apparent from the trait means summarized in Table 1. Grasslands Huia had almost 120% the stolon length and 140% the number of apexes of the other two entries, but the stolon DW, leaf DW, and root DW were approximately 62, 62, and 75%, respectively, of the average weights of the other entries. The cumulative effects of these differences resulted in Grasslands Huia having 35% less yield and biomass than the other entries. The leaf-to-stem ratio was similar for all entries but the herbage-to-root ratio varied from 3.22 to 4.09.

Evaluating the white clover at three times during the year exposed plants to very different environments (Table 2). The spring evaluation, which ended on 6 June, produced biomass about four times that of the summer and winter tests. It is speculated that cooler temperatures, particularly at the beginning of the test, and longer days promoted greater growth. Spring stolon lengths and stolon weights were 3.6 and 5 times greater, respectively, than the average of the other two seasons and the DW of leaf, root, and herbage were 3.5 to 4.2 times greater in the spring evaluation. The leaf-to-stem ratio for the spring season was lower at 1.16 versus 1.54 and 1.79 for the other seasons. The herbage-to-root ratio varied only from 3.61 to 3.83 and the apex number varied from 28.5 to 80.8 across seasons. Seasonal environment greatly affected morphology and average performance and altered energy partitioning in the plant as measured by the leaf-to-stem and herbage-to-root ratios.

Except for the ratios, the effect of clipping was to reduce each measurement by at least 40% (Table 3). The differences between clipped and not clipped were highly significant for all traits including the ratios (Table 4). The rooting barrier significantly affected stolon DW, apex number, root DW, leaf-to-stem ratio, and herbage-to-root ratio. The interactions of rooting barrier and clipping for apex number, root DW, and herbage-to-root ratio suggest the effect of the rooting barrier was not proportional for each clipping treatment.

Clipping caused reductions of 50% for stolon length, 75% for stolon weight, 88% for leaf weight, and 80% for root weight (Table 3). The cumulative effects of the clipping treatments were an approximate 85% decrease in herbage and biomass DW. With clipping, the leaf-to-stem ratio was about one-third that of the unclipped treatments (0.65 vs. 1.80) which reflects reduced leaf production in comparison to retained stolon weight. The rooting barrier significantly increased the stolon DW, decreased the root DW, decreased the leaf-to-stem ratio, and increased the herbage-to-root ratio (Tables 3 and 4).

Treatments had some very large effects on DWs, apex number, and the ratios, but it is not known if all plants responded similarly to these stresses. Consistency in clone responses is shown in the correlations between treatments for each trait (Table 5). These correlations never exceeded 0.51 which were statistically significant but of limited biological significance ($r^2 \leq 0.25$). Thus, relative performance of a clone in one of the four treatments did not accurately predict its relative response in another treatment. In particular, no correlation was significant for the treatment of plants with rooting barrier and not clipped.

Path analysis, unlike a table of means, generates an estimate of the change in SDs of the dependent variable in terms of 1 SD change of each independent variable and estimates correlated responses between independent variables. The absence of biologically significant correlations between treatments (Table 5) motivated a separate path analysis for each combination of treatments. The models fit were (i) leaf DW as dependent variables with independent variables root DW, apex number, stolon length, and stolon DW, (ii) root DW as dependent variable with independent variables apex number, stolon DW, and stolon length, and (iii) herbage DW as dependent variable with independent variables root DW, stolon length, and apex number.

For leaf DW, two direct effects, stolon length and apex number, were small or not significantly different from zero for all treatments (Fig. 1). The direct effects of stolon DW were ≥ 0.60 without clipping, but when

Table 2. Trait means of individual plants for white clover tested in spring, summer, and winter.

Trait	Summer of test		
	Spring (2 April–6 June)	Summer (7 June–14 September)	Winter (22 October–2 February)
Stolon length (cm)	991	240	299
Stolon DW (g)	11.88	2.48	2.27
Apex number	80.8	28.5	53.3
Leaf DW (g)	13.76	3.81	4.06
Root DW (g)	7.07	1.74	1.65
Herbage DW (g)	25.64	6.29	6.32
Biomass DW (g)	32.69	8.02	7.97
Leaf-to-stem ratio	1.16	1.54	1.79
Herbage-to-root ratio	3.62	3.61	3.83

Table 3. Trait means of individual plants exposed to treatment combinations of with and without clipping on 7-d intervals and the presence or absence of a barrier to stolon rooting.

	Treatment			
Trait	Plants clipped Rooting barrier	Plants clipped No barrier	Plants not clipped Rooting barrier	Plants not clipped No barrier
Stolon length (cm)	344	312	704	680
Stolon DW (g)	2.41	1.96	9.32	8.48
Apex number	37.8	36.9	64.8	77.4
Leaf DW (g)	1.50	1.55	12.96	12.81
Root DW (g)	1.10	1.16	5.02	6.61
Herbage DW (g)	3.92	3.50	22.28	21.30
Biomass DW(g)	5.02	4.66	27.30	27.9
Leaf-to-stem ratio	0.61	0.73	1.76	1.92
Herbage-to-root ratio	3.35	2.91	5.08	3.54

Table 4. Analysis of variance on nine traits of random white clover plants from two cultivars and germplasm tested in greenhouse in three different seasons (spring, summer, and winter) during a year.

Source	Stolon length	Stolon DW	Apex number	Leaf DW	Root DW	Herbage DW	Biomass DW	Leaf-to-stem ratio	Herbage-to-root ratio
Season(SES)	–	–	–	–	–	–	–	–	–
Cultivar	**	**	**	**	**	**	**	ns	**
SES×cultivar	ns	**	**	**	**	**	**	ns	**
Rep(SES)	–	–	–	–	–	–	–	–	–
Clipping	**	**	**	**	**	**	**	**	**
Rooting barrier	ns	*	**	ns	**	ns	ns	**	**
Clipping×barrier	ns	ns	**	ns	**	ns	ns	ns	**

* Indicates significance of main effect or interaction at $\alpha = 0.05$.
** Indicates significance of main effect or interaction at $\alpha = 0.01$.
ns, is not significant.

Table 5. Correlations for same trait measured on the ramets of each clone exposed to treatments of no clipping or clipping on 7-d intervals in combination with a treatment of a barrier to stolon rooting or no barrier.

		Treatments		
Treatment	Traits	Plants clipped No rooting barrier	Plants not clipped Rooting barrier	Plants not clipped No rooting barrier
Plants clipped Rooting barrier				
	Stolon length	0.40*	−0.06	0.25*
	Stolon DW	0.37*	−0.09	0.37*
	Apex number	0.35*	0.02	0.29*
	Leaf DW	0.21*	0.00	0.25*
	Root DW	0.30*	−0.06	0.16
	Herbage DW	0.36*	−0.05	0.33*
	Biomass DW	0.35*	−0.06	0.29*
	Leaf-to-stem ratio	0.05	−0.02	0.16
	Herbage-to-root ratio	0.34*	−0.02	0.17
Plants clipped No rooting barrier				
	Stolon length		0.07	0.37*
	Stolon DW		−0.09	0.51*
	Apex number		0.09	0.25*
	Leaf DW		−0.02	0.25*
	Root DW		−0.03	0.30*
	Herbage DW		−0.07	0.43*
	Biomass DW		−0.08	0.42*
	Leaf-to-stem ratio		−0.04	0.11
	Herbage-to-root ratio		−0.00	0.06
Plants not clipped Rooting barrier				
	Stolon length			0.05
	Stolon DW			0.00
	Apex number			0.00
	Leaf DW			0.00
	Root DW			0.04
	Herbage DW			−0.00
	Biomass DW			−0.01
	Leaf-to-stem ratio			−0.06
	Herbage-to-root ratio			0.03

* Indicates correlation coefficient statistically significant from zero at $\alpha = 0.05$.

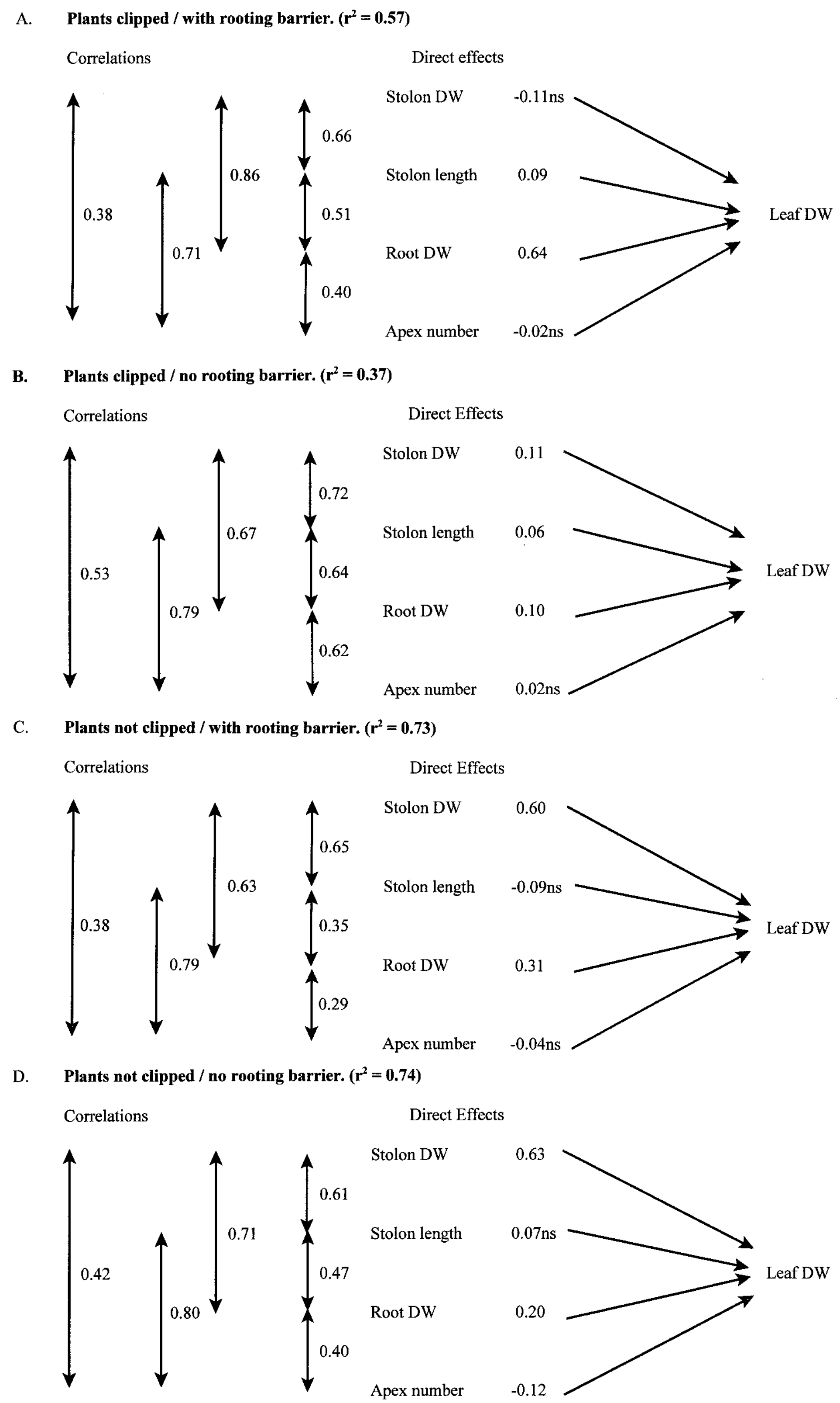

Fig. 1. Path analysis on leaf dry weight with direct effects of stolon DW, stolon length, root DW, and apex number and their indirect effects (correlations). Separate estimates were made for each of the four management treatments. ns Indicates that direct effect coefficients are not significantly different from zero at α = 0.05 and unmarked coefficients are significant.

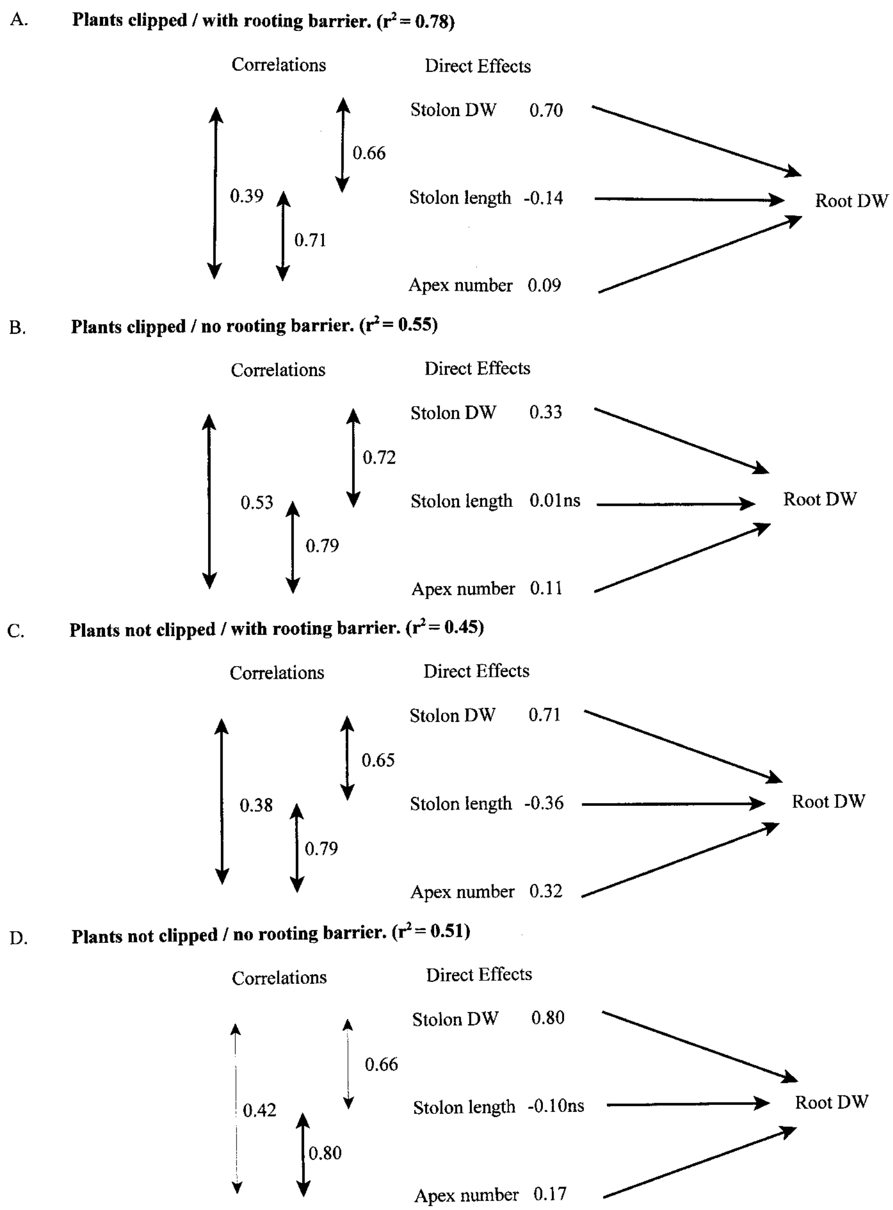

Fig. 2. Path analysis on root DW with direct effects of stolon DW, stolon length, and apex number and their indirect effects (correlations). Separate estimates were made for each of the four management treatments. ns Indicates that direct effect coefficients are not significantly different from zero at $\alpha = 0.05$ and unmarked coefficients are significant.

the plants were clipped the coefficient of direct effect stolon DW approached zero. The effect of rooting barrier did not have a consistent effect on direct effect of root DW. In absence of a rooting barrier, the coefficient was 0.10 or 0.20 (Fig. 1, A and B); when plants were not clipped and had a rooting barrier, the coefficient was 0.31 (Fig. 1, C); and when plants were clipped and had a rooting barrier, the coefficient increased to 0.64 (Fig. 1, A).

For the root DW, the coefficients for direct effects (Fig. 2) did not follow a consistent pattern attributable to either treatment; but rather, reflect the significant interaction between treatments (Table 4). The path coefficients for direct effect of stolon length and apex number were −0.36 and 0.32, respectively, when plants had a rooting barrier and were not clipped (Fig. 2, C), but for the other three treatments the path coefficients were near zero (Fig. 2, A, B, and D). In contrast, the direct effect for stolon DW was 0.33 when plants were clipped and did not have a rooting barrier, but for the other treatments the direct effect coefficients were ≥ 0.70.

For herbage DW, clipping greatly affected the direct effect of apex number and stolon length (Fig. 3). All path coefficients for these two direct effects were significantly different from zero, but when plants were clipped, coefficients were one third or less of the estimates for plants not clipped. The direct effects on root DW varied for each treatment but was smallest without a rooting barrier (values of 0.32 and 0.49).

CONCLUSIONS

In replicated greenhouse testing over time of genetically variable white clovers, the stress of frequent clip-

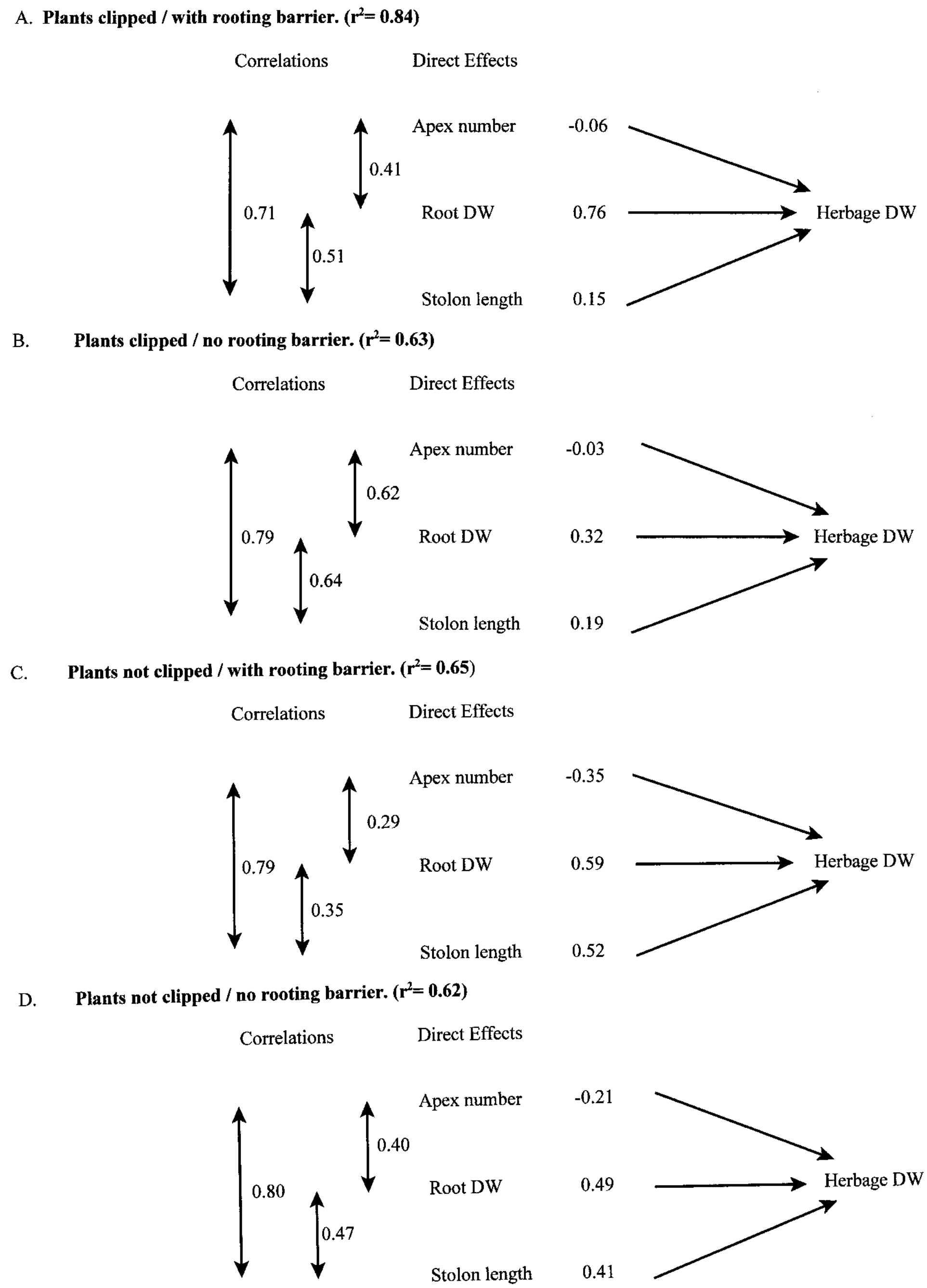

Fig. 3. Path analysis on herbage DW with direct effects of apex number, root DW, and stolon length and their indirect effects (correlations). Separate estimates were made for each of the four management treatments. ns Indicates that direct effect coefficients are not significantly different from zero at α = 0.05 and unmarked coefficients are significant.

ping significantly reduced all plant parameters and the leaf-to-stem ratio and the herbage-to-root ratio. The barrier to stolon root development had a less dramatic effect but significantly reduced apex number and root DW and increased stolon DW. The results were significant decrease in the leaf-to-stem ratio and significant increase in the herbage-to-root ratio.

Though plasticity is commonly observed as the changes in leaf-to-stem ratio and plant size, in this study, the plasticity was quantified by the changes in path analysis estimates of direct effects and indirect effects on dependent varaibles root DW, leaf DW, and herbage DW. The estimated direct effects varied for each of the stress treatment combinations. The degree of plasticity varied among the clones and caused near zero correlations for the same measurement made on the same clone grown with and without the treatment stresses.

Plasticity is a characteristic of the plant, which is thought to improve the plant persistence in presence of interspecific competition or with grazing pressure, but it is likely to hinder the selection for improved genotypes. Verification that progenies of superior selections are also superior in an array of environments with different stresses and growing conditions appears difficult. If plasticity is a heritable characteristic that is separable from other genetic factors affecting yield and persistence, then cultivar improvement based on consistent performance in multiple environments in cultivar evaluation trials could be selection for reduced plasticity with unintended and undesirable effects on white clover adaptability and persistence.

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